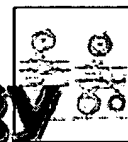




# Diagnostic Molecular Pathology



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Diagnostic Molecular Pathology: Volume 9(1) March 2000 pp 47-57

## Correlative Immunohistochemical and Reverse Transcriptase Polymerase Chain Reaction Analysis of Somatostatin Receptor Type 2 in Neuroendocrine Tumors of the Lung [Original Articles]

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### Abstract TOP

Somatostatin receptors type 2 (sst2) have been frequently detected in neuroendocrine tumors and bind somatostatin analogues, such as octreotide, with high affinity. Receptor autoradiography, specific mRNA detection and, more recently, anti-sst2 polyclonal antibodies are currently employed to reveal sst2. The aim of the present study was to investigate by three different techniques the presence of sst2 in a series of 26 neuroendocrine tumors of the lung in which fresh frozen tissue and paraffin sections were available. It was possible, therefore, to compare, in individual cases, RNA analysis studied by reverse transcriptase polymerase chain reaction (RT-PCR), in situ hybridization (ISH), and immunohistochemistry. A series of 20 nonneuroendocrine lung carcinoma samples served as controls. RT-PCR was positive for sst2 in 22 of 26 samples, including 15 of 15 typical carcinoids, 5 of 6 atypical carcinoids, and 2 of 5 small-cell carcinomas. The sst2 mRNA signal obtained by RT-PCR was strong in the majority (87%) of typical carcinoids and of variable intensity in atypical carcinoids and small-cell carcinomas. A weakly positive signal was observed in 5 of 20 control samples. In immunohistochemistry, two different antibodies (anti-sst2) were employed, including a monoclonal antibody, generated in the Department of Pathology, University of Turin. In the majority of samples a good correlation between sst2 mRNA (as detected by RT-PCR) and sst2 protein expression (as detected by immunohistochemistry) was observed. However, one atypical carcinoid and one small-cell carcinoma had focal immunostaining but no RT-PCR signal. ISH performed in selected samples paralleled the results obtained with the

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other techniques. A low sst2 expression was associated with high grade neuroendocrine tumors and with aggressive behavior. It is concluded that 1) neuroendocrine tumors of the lung express sst2, and there is a correlation between the mRNA amount and the degree of differentiation; 2) immunohistochemistry and ISH are reliable tools to demonstrate sst2 in these tumors; and 3) sst2 identification in tissue sections may provide information on the diagnostic or therapeutic usefulness of somatostatin analogues in individual patients with neuroendocrine tumors.

The somatostatin receptor family (sst) includes at least five isoforms that have been recently identified and characterized (18,32,41). The ssts are widely distributed in normal human tissues and in human tumors. Sst type 2 is more commonly detected in neuroendocrine tumors (32,37) and binds the somatostatin analogue octreotide with high affinity.

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Sst localization had originally been demonstrated by means of binding assays of radiolabeled somatostatin analogues (20,25,31). Subsequently, specific sst messenger RNA (mRNA) detection was obtained by means of in situ hybridization (ISH) and reverse transcriptase polymerase chain reaction (RT-PCR) (14,32,37). Recently, polyclonal antibodies specific for different isoforms of sst were produced and used in immunohistochemistry (10,12,15,18,30,35,36). Given the well-known heterogeneity of neoplastic populations, in situ methods (immunohistochemistry and ISH) allow a more definite mapping of the distribution of the receptor in such tissues. This is potentially useful for predicting the responsiveness of a given neoplastic cell population to medical treatment with somatostatin analogues, which are used in the clinical setting for both diagnostic and therapeutic purposes with special reference to neuroendocrine tumors.

The spectrum of neuroendocrine tumors of the lung includes well-differentiated neoplasms (so-called typical carcinoids) and poorly differentiated small-cell carcinomas (SCCs). Intermediate forms sharing features of both the aforementioned types also belong to this spectrum (so-called atypical carcinoids or well-differentiated neuroendocrine carcinomas). Finally, large-cell neuroendocrine carcinoma has been identified and included in this tumor group (4,40). The tissue distribution of sst2 in neuroendocrine tumors of the lung has not been thoroughly characterized, although individual samples of bronchial carcinoids were found to express sst (30). SCCs (but not non-small-cell types) were also shown to be sst2 positive by receptor binding assay (33). Moreover, sst2 has been detected in in vitro cell cultures of human SSC of the lung (39,42). No study on a series of neuroendocrine tumors of the lung including all neuroendocrine lung tumor types has been reported to date.

The aim of this study was therefore to investigate the presence of sst2 mRNA and protein in a series of 26 neuroendocrine tumors of the lung, employing different technical approaches, such as RT-PCR, ISH, and immunohistochemistry. To this purpose a monoclonal antibody to sst2 (N-terminal) was generated in the Department of Pathology, University of Turin. The results were then compared and related to the tumor grade and to other clinicopathologic parameters.

## MATERIALS AND METHODS TOP

### Case Series and RNA Extraction TOP

Twenty-six samples of neuroendocrine tumors of the lung, in which fresh frozen tissue was available, were retrieved from the surgical pathology file of the University of Turin, Italy. All samples were reviewed applying currently accepted criteria of classification (4,40), and the neuroendocrine nature was confirmed by positive immunostaining for chromogranin A (CgA) (with or without antigen retrieval) or synaptophysin, and by positive RT-PCR for CgA mRNA. According to the classifications described here, these included 15 well-differentiated neuroendocrine tumors (typical carcinoids), 6 well-differentiated neuroendocrine carcinomas (atypical carcinoids), and 5 SCCs.

A series of 20 non-small-cell lung carcinomas (10 squamous, 9 adenocarcinomas, and 1 large-cell anaplastic) lacking neuroendocrine differentiation, as demonstrated by negative immunohistochemistry and RT-PCR for CgA (1), served as a control group. Clinicopathologic data and follow-up information were obtained for all patients.

For hybridization analysis, total RNA was extracted using the guanidine thiocyanate-cesium chloride method (5). The concentration of RNA was estimated by spectrophotometry, and RNA degradation was assessed by agarose gel electrophoresis, as previously reported (37).

**Reverse Transcriptase Polymerase Chain Reaction for sst2 and Chromogranin A <sup>top</sup>**

Total RNA (2 µg) was first digested, with 10 units of RNase-free DNase (Boehringer, Mannheim, Germany) in a 10-µL solution containing 20 mmol/L MgCl<sub>2</sub>, to avoid DNA contamination. The solution was kept at room temperature for 10 minutes, then heated for 5 minutes at 70°C to inactivate the DNase molecules; 40 pmol/L of oligodeoxythymidine primers (oligo-dT16) were added and the solution was heated again at 70°C for 10 minutes, then chilled on ice to allow the primer hybridization. The resulting solution was reverse transcribed using 100 units of reverse transcriptase (Gibco BRL, Gaithersburg, MD). Complementary DNA (cDNA) was generated in a 50-µL final reaction volume containing 50 mmol/L Tris-HCl pH 8.3, 75 mmol/L KCl, 3 mmol/L MgCl<sub>2</sub>, 10 mmol/L dithiothreitol, 1 mmol/L deoxynucleotide triphosphates (dNTPs), and 20 units of RNasin (Promega, Madison, WI). The solution was heated at 37°C for 90 minutes. Finally, the enzymes were inactivated by heating to 70°C for 10 minutes.

The efficiency of the reverse transcription was determined by performing a PCR reaction having the  $\beta_2$ -microglobulin housekeeping gene as a target. PCR was carried out in a 10-µL final reaction volume containing 1 µL of cDNA template, 10 pmol of sense and antisense oligonucleotide primers, 67 mmol/L Tris-HCl pH 8.8, 16 mmol/L (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.01% polysorbate 20, 2 mmol/L dNTPs, 1 mmol/L MgCl<sub>2</sub>, and 0.5 units of Taq polymerase.  $\beta_2$ -Microglobulin, sst2, and CgA PCR reactions were performed using the same protocol at the following PCR conditions: 35 cycles, each cycle consisting of denaturation at 94°C for 2 minutes, annealing at 55°C for 1 minute for  $\beta_2$ -microglobulin, at 61°C for sst2, and at 68°C for CgA; extension was performed at 72°C for 1 minute. The primers used for RT-PCR (9,11,23,37) are reported in Table 1.

- 
- 
- 1)  $\beta_2$ -microglobulin sense: 5' ACC CCC ACT GAA AAA GAT GA 3'
  - 2)  $\beta_2$ -microglobulin antisense: 5' ATC TTC AAA CCT CCA TGA TC
  - 3) SSTR2 sense: 5' CAG TCA TGA GCA TCG ACC GA 3'
  - 4) SSTR2 antisense: 5' GCA AAG ACA GAT GAT GGT GA 3'
  - 5) CgA sense: 5' GCT CCA AGA CCT CGC TCT CC 3'
  - 6) CgA antisense: 5' GAC CGA CTC TCG CCT TTC CG 3'
- 

PCR, polymerase chain reaction.

**TABLE 1.** Sequences of primers used for reverse transcriptase polymerase chain reactionPCR, polymerase chain reac

The amplified fragments were run in a 1% agarose gel, containing ethidium bromide. Strict precautions against contamination were undertaken (19) and negative controls (a no-template control and a no-reverse transcriptase control and distilled water to replace the RNA) were included. The RNA extracted from an H716 neuroendocrine colon carcinoma cell line and from a neuroblastoma (37) served as positive controls for CgA and sst2, respectively.

**Antibodies <sup>top</sup>**

Two different antibodies specific for sst2 were employed. The first one was a monoclonal antibody raised in the Department of Pathology (University of Turin) specific for an N-terminal sequence of the sst2 (shared by both A and B receptor isoforms). The octapeptide EPYYDLTS, corresponding to amino acids 35 to 42 of the human receptor (and differing by one amino acid from the mouse sequence), was synthesized, having a lysin added to the N-terminal. This sequence was similar to that used by other groups to produce polyclonal antibodies (17,18,27). This sequence was rather short but made it possible to avoid extensive homology with sst1. In addition, according to a genbank search using FASTA (28), this protein sequence is unique to human sst2 and has a partial homology only with rat and human nuclear receptor retinoid orphan nuclear receptor-beta (a protein having nuclear localization). Three Balb/c mice were immunized with the peptide conjugated to keyhole limpets hemocyanin (KLH) (Sigma, St. Louis, MO) following the standard procedure. After the first intrasplenic injection (100 µg of protein) at time 0, the

mice were intraperitoneally injected six times with the peptide-KLH conjugate (150 µg) in the presence of Freund adjuvant. The reactivity of the sera from each animal was evaluated using an enzyme-linked immunosorbent assay, using the peptide coated onto the plastic. The hybridomas were produced by somatic fusion of immunized splenocytes with the mouse myeloma cell line Ag8.X63.653, following the standard technique (21). The monoclonal antibodies of interest were selected on the basis of the reactivity with the target peptide and with appropriate tissue sections. The latter included formalin-fixed and paraffin-embedded sections of pituitary gland and pancreatic islets and were analyzed by means of immunoperoxidase staining. Parallel control experiments were also performed by staining serial sections of these tissues, omitting the primary antibody or with the preimmune serum or with the antibody preadsorbed with high concentrations (1 mg/mL) of the antigenic peptide. In addition, the selected monoclonal antibodies (coded 10C6 and 10G4), both of IgM isotype, were further characterized by Western blotting. Membranes were prepared from stable transfected Chinese hamster ovary (CHO)-K1 cells, individually expressing recombinant human somatostatin receptors (sst1 to sst5). Western blotting was performed as previously described (36). The monoclonal antibody was used as culture supernatant at 1:3 dilution for 2 hours at room temperature in Tris-buffered saline (TBS), supplemented with 0.1% polysorbate 20. Blots were washed in TTBS and incubated with peroxidase-conjugated goat antimouse IgM, diluted 1:1.500 for 90 minutes at room temperature. Then, blots were washed in TTBS and immunocomplexes were visualized using ECL following manufacturer's instructions (Amersham, Bucks, UK).

A second polyclonal antibody was produced that had been characterized previously (35,36). This antibody (coded K230) was raised in sheep and was specific for a sequence of the C-terminal portion of the sst2A (KSRLNETTETQRTLLNEDLQ, amino acids 347 to 366).

### Immunohistochemistry TOP

Sections 4 or 5 µ thick, adjacent to those used for conventional histopathologic examination and immunostaining for neuroendocrine markers, were collected onto poly-L-lysine-coated slides. The proliferative activity of the tumors was assessed by means of Ki67 immunostaining (clone MIB1, Immunotech, Marseille, France), diluted 1:10 after microwave-based antigen retrieval in citrate buffer). The ascitic fluid of monoclonal antibody 10G4 was used in this study and was applied to tissue sections with prior antigen retrieval (three 3-minute passages in a microwave oven at 800 W in citrate buffer pH 6.0), at the dilution of 1:10,000 or 1:12,000 for 30 minutes at room temperature. The antiserum coded K230 was applied overnight at a dilution of 1:300 with no prior antigen retrieval. The immune reactions were then revealed with the immunoperoxidase technique (13) using the streptavidin-peroxidase kit and diaminobenzidine as chromogen. A weak nuclear counterstain or no counterstain was used in parallel sections. Control stainings for both antibodies included immunoperoxidase of serial sections using preimmune serum or antibody preadsorbed with the antigen or buffer instead of the primary antibody.

### *In Situ Hybridization* TOP

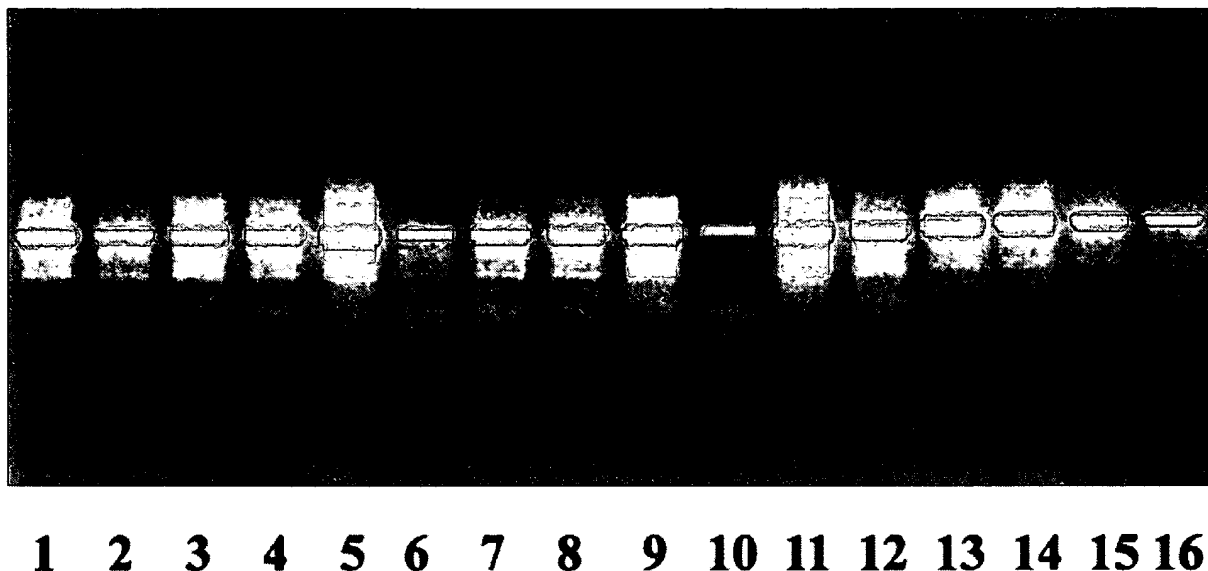
Selected tumors (12 samples) were also analyzed for sst2 mRNA expression by means of a nonradioactive, tyramide deposition-based ISH technique. The procedure of amplification was modified from procedures reported by Kerstens et al. (16), Speel et al. (38), and the GenPoint (biotinyl-tyramide) manufacturer (Dako, Glostrup, Denmark). Briefly, 5-µm-thick paraffin sections were collected onto silane-coated slides and deparaffinized through xylene and graded alcohols to phosphate buffer saline (PBS). The slides were then incubated for 5 minutes in a microwave oven at 800 W in citrate buffer pH 6.0. After washing in PBS, they were digested with proteinase K (1 µg/mL) for 10 minutes at 23°C. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide and endogenous biotin was blocked using avidin-blocking reagent for 15 minutes followed by washing in PBS and biotin-blocking reagent for 15 minutes (3). Sections were then prehybridized for 1 hour at room temperature in a mixture composed of 4× SSC, 50% formamide, Denhardt's 1×, dextrane sulfate 5×, 500 µg/mL salmon sperm DNA, and 250 µg/mL tRNA. Hybridization took place overnight at 42°C in a solution containing the specific probe at a concentration of 1 pmol/mL. The probe was a digoxigenin-labeled 48-base oligonucleotide (32), complementary to positions 91 to 139 of the human sst2 gene (41). After hybridization, excess hybridization buffer and coverslips were removed by a rapid wash in 4× SSC followed by stringent washing in 0.1× SSC for 10 minutes at 42°C. The hybrids were revealed by the following incubation steps: peroxidase-labeled antidigoxigenin (diluted 1:100 in PBS) for 30 minutes at room temperature, biotinylated tyramide (diluted 1:5 in PBS) for 15 minutes at room temperature, and peroxidase-labeled streptavidin for 15 minutes at room temperature. Diaminobenzidine was used as chromogen. Controls for ISH included staining of serial sections with sense probe, an unrelated probe (EBER-1 of the Epstein-Barr virus), and omission of the probe in the hybridization mixture, with all other experimental conditions identical to the procedure described here.

## RESULTS TOP

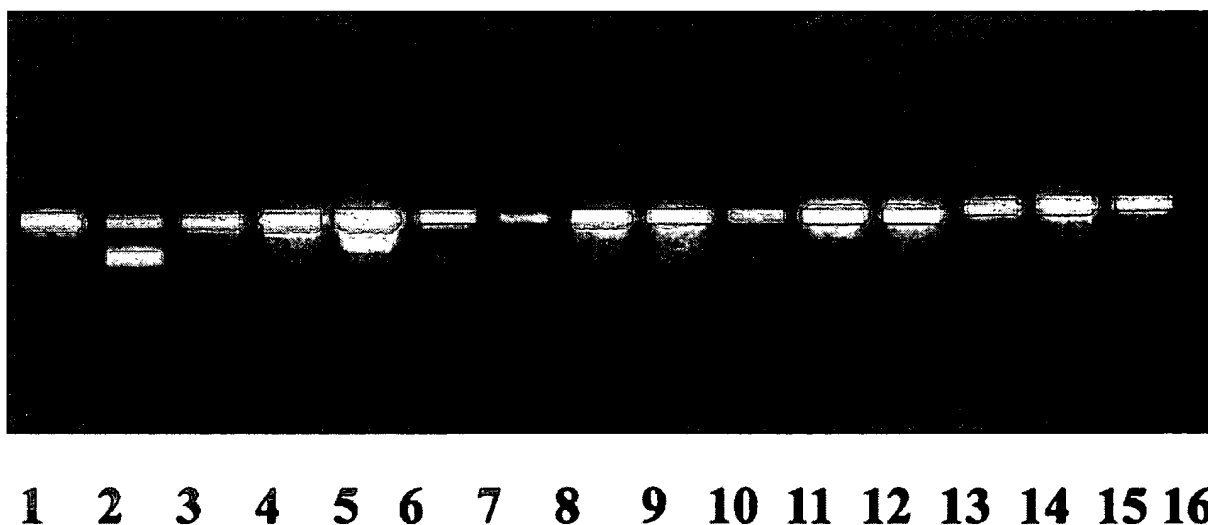
### Reverse Transcriptase Polymerase Chain Reaction TOP

All neuroendocrine tumors, but no nonneuroendocrine lung carcinomas, were positive for CgA mRNA (Fig. 1). Sst2

mRNA was amplified in 22 of 26 samples of neuroendocrine tumor. The signals had variable intensities (Fig. 2) and were weak in moderately or poorly differentiated tumors (mostly in SCCs). No amplification was obtained in no-template or no-reverse transcriptase experiments. Control samples (nonneuroendocrine lung carcinomas proven by negative CgA RT-PCR) were weakly positive for sst2 in 5 of 20 samples only (including 3 adenocarcinomas, 1 squamous, and the large-cell anaplastic carcinoma) (Fig. 3). These differences were statistically significant ( $P < 0.01$ ) by  $\chi^2$  test.



**FIG. 1.** Reverse transcriptase polymerase chain reaction for chromogranin A (CGA) mRNA in 26 samples of neuroendocrine tumor. Lanes 1-16 show a variable intensity of the amplification band. C and C\* stand for positive (neuroendocrine colon carcinoma cell line, H716) and negative (distilled water) controls, respectively.



**FIG. 2.** Reverse transcriptase polymerase chain reaction for sst2 mRNA in 26 samples of neuroendocrine tumor of the lung. Lanes 1-16 show a variable intensity of the amplification band. C and C\* stand for positive (a neuroblastoma) and negative (distilled water) controls, respectively. The last column to the right represents the amplification band.



**FIG. 3.** Reverse transcriptase polymerase chain reaction for sst2 mRNA in 20 control samples of nonneuroendocrine lu and C\*) are identical to those in [Fig. 2](#).

Patient no.	Diagnosis	Sex/age	Size (cm)	Follow-up (mo)	CgA IHC
1	WD NET	F/35	3.5	NED 90	+
2	WD NET	F/29	4	NED 45	+
3	WD NET	F/41	2.5	NED 70	+
4	WD NET†	F/25	4	NED 21	+
5	WD NET†	M/58	3.8	NED 23	+
6	WD NET	M/52	2.5	NED 42	+
7	WD NET	F/69	3.5	NED 47	+
8	WD NET	M/29	3	NED 70	+
9	WD NET	M/27	4	NED 108	+
10	WD NE Ca‡	M/66	8	AWD 55	+
11	WD NET‡	F/29	2	AWD 56	+
12	WD NET*	F/32	3	NED 26	+
13	WD NE Ca	M/60	3	NED 133	+
14	WD NET	M/28	4	NED 130	+
15	WD NET‡	M/41	1.3	AWD 53	+
16	WD NET†	F/31	1	NED 13	+
17	WD NET	F/53	4	NED 24	+
18	WD NE Ca	M/62	3	NED 6	-
19	WD NE CA	F/73	5	DOD 20	+
20	SCC	M/57	6	DOD 12	+
21	SCC	M/51	4.5	DOD 5	+
22	WD NE Ca	M/60	6	NED 51	+
23	SCC	F/56	6	DOD 11	+
24	WD NE Ca	M/77	2.5	NED 21	+
25	SCC	M/57	5	DOD 10	+
26	SCC†	M/68	11	recent case	+

AWD, alive with disease; CgA, chromogranin A; DOD, died of disease; Mab, monoclonal antibody; NECa, Neuroendocrine carcinoma; NED, no evidence of disease; RT-PCR, reverse transcriptase polymerase chain reaction; SCC, Small-cell lung carcinoma; SYP, synaptophysin; WD, well differentiated.

\* Ki67 IHC: values correspond to percentage of positive nuclei of

† Patients who had preoperative octreoscan performed.

‡ Patients who had octreoscan performed and octreotide treatment.

**TABLE 2.** Clinicopathologic data and somatostatin receptor type 2 (*sst2*) expression in 26 cases of neuroendocrine lung cancer. IHC, Immunohistochemistry; Mab, monoclonal antibody; NECa, Neuroendocrine carcinoma; NED, no evidence of disease; RT-PCR, reverse transcriptase polymerase chain reaction; SCC, Small-cell lung carcinoma; SYP, synaptophysin; WD, well differentiated. \*Ki67 IHC: values correspond to percentage of

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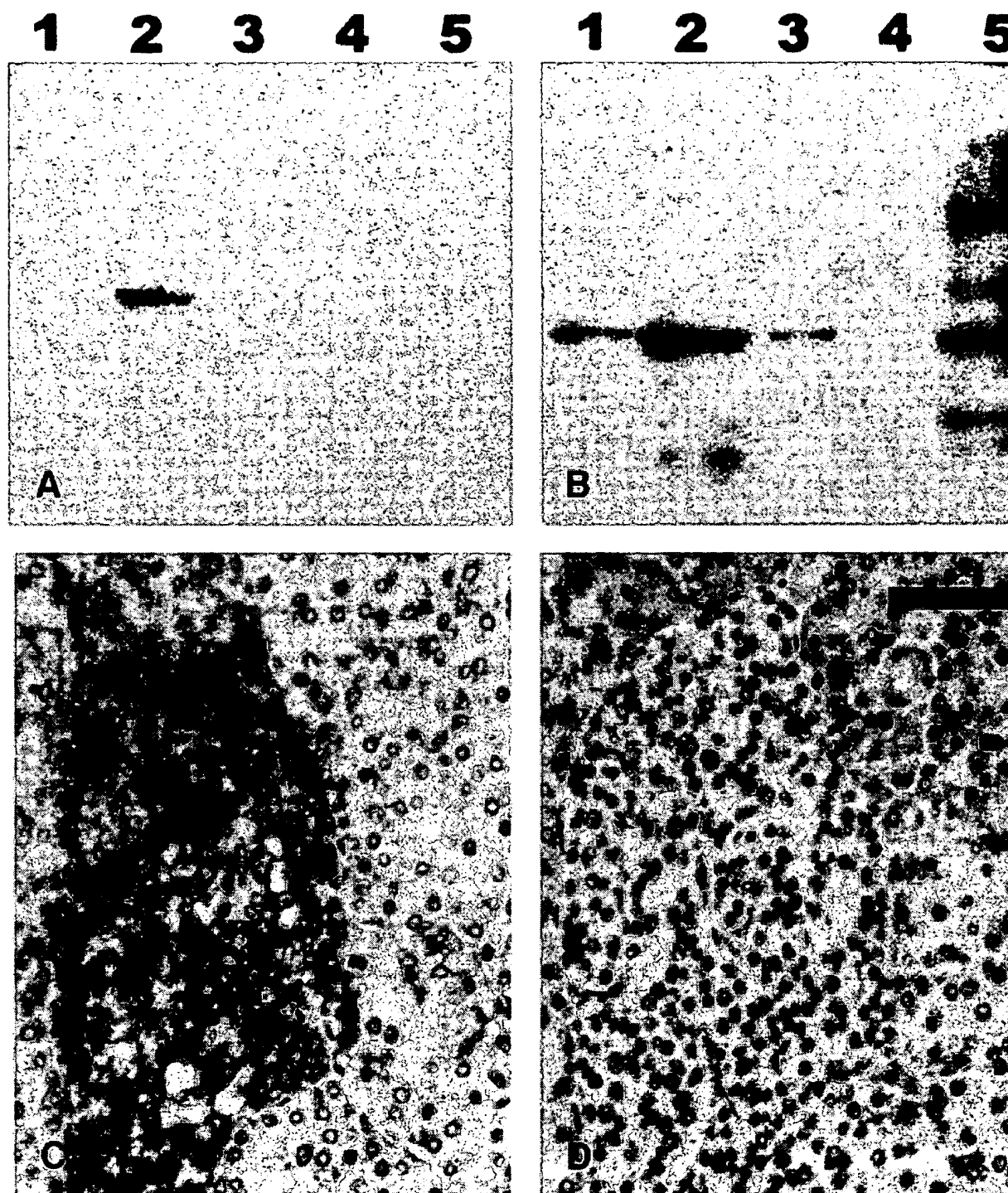
octreoscan performed and octreotide treatment.

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### Characterization of Monoclonal Antibodies to sst2 <sup>TOP</sup>

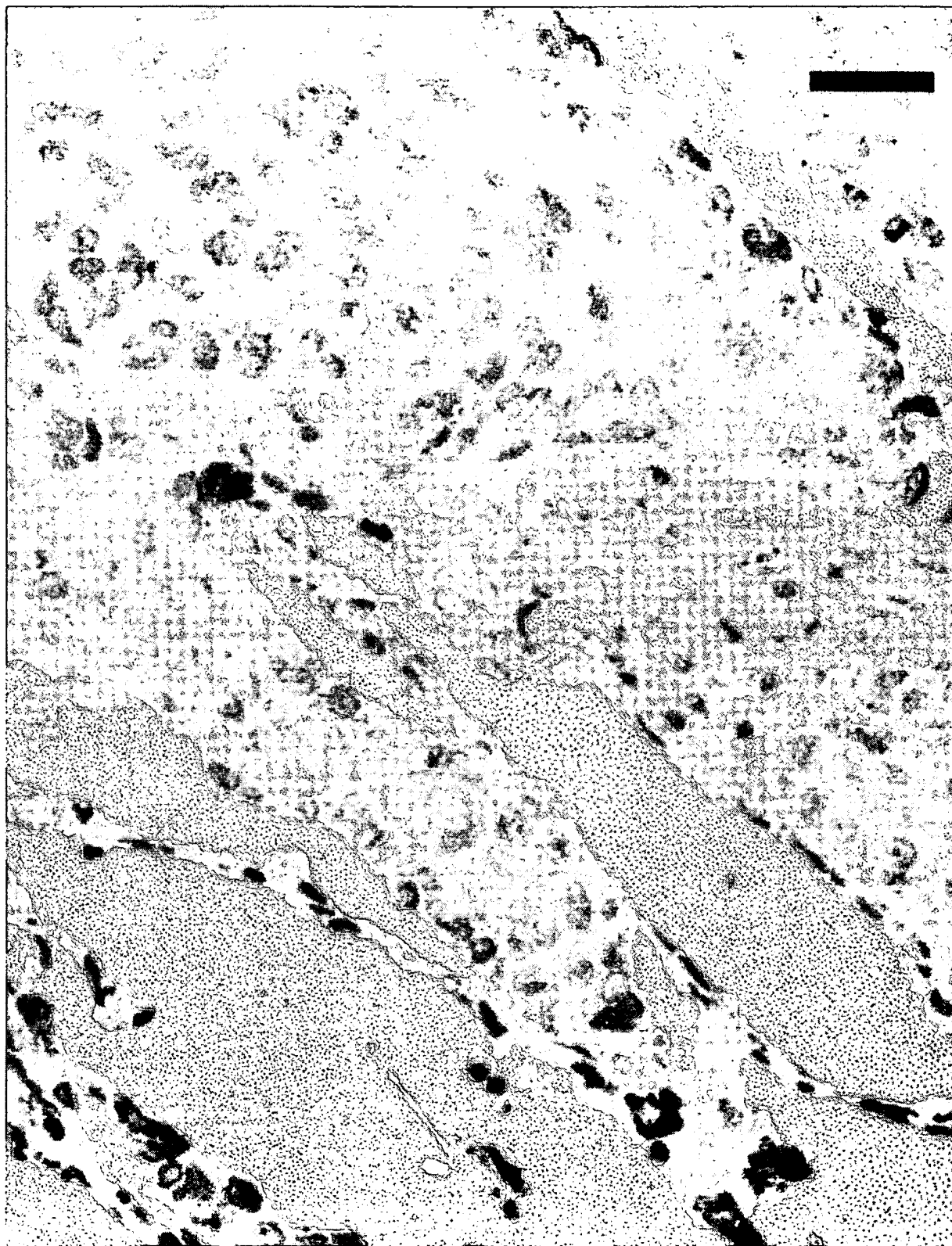
Several clones were identified having a positive binding by enzyme-linked immunosorbent assay and a parallel immunoreactivity on formalin-fixed paraffin-embedded human endocrine tissues (pituitary and pancreatic islets). In Western blotting experiments, two clones (coded 10C6 and 10G4) specifically developed a band at approximately 70 kD. When the antibodies were used against CHO-transfected cells expressing recombinant somatostatin receptors 1 through 5, a specific band corresponding to sst2 (at approximately 70 kD) was revealed by the monoclonal antibody 10G4. Monoclonal antibody 10C6 developed a strong band with sst2 but displayed a weaker reactivity also with sst1, 3, and 5, at least in the present experimental conditions (**Fig. 4 A,B**). The same antibodies were also tested by means of immunoperoxidase staining on formalin-fixed, paraffin-embedded samples of normal human pituitary gland and pancreas. Monoclonal antibody 10G4 gave good results in immunohistochemistry and was used at increasing dilutions (up to 1:15,000) with specific staining. Using thin sections (approximately 4  $\mu$ m), a strong membrane-bound and peripheral cytoplasmic immunoreactivity was found in an adenohypophyseal cell population (corresponding to growth hormone-secreting cells, as confirmed by double immunohistochemical analyses) and in pancreatic islets (**Fig. 4 C,D**). In the latter, the staining was apparently not restricted to a specific hormone-producing cell type and had a peripheral cytoplasmic or membrane distribution. Exocrine pancreatic cells (both acinar and ductal) were only occasionally immunostained. Immunohistochemistry performed on serial control sections, either omitting the primary antibody or using the preimmune serum or antibodies preabsorbed with the synthetic peptide, was negative in both tissues. Monoclonal antibody 10C6 had a relatively higher background staining at similar dilutions.





**FIG. 4.** Western blot analysis of monoclonal antibody clones 10G4 and 10C6 against sst2 in Chinese hamster ovary cell transfected with recombinant sst 1 through 5 (numbers of each column correspond to receptor type). Monoclonal antibody 10G4 shows a specific band at approximately 70 kD for sst2 only (A) as opposed to monoclonal antibody 10C6, which strongly reacts with sst2 but also has some degrees of cross-reactivity with sst 1, 3, and 5 (B). The lower figures show control formalin-fixed paraffin-embedded pancreatic islets immunostained with monoclonal antibody 10G4 without (C) and with (D) preadsorption with the peptide antigen, respectively. The majority of endocrine cells show a membrane-bound immunoreactivity (C) (immunoperoxidase). Bar: 90  $\mu$ m.

The antibodies to sst2 (monoclonal antibody 10G4 and polyclonal K230) gave slightly different immunoreactions in 25 samples, and staining was not done in 1 sample because of lack of residual paraffin blocks. The monoclonal antibody 10G4 stained 21 of 25 samples, the negative samples being 1 atypical carcinoid and 3 SCCs (Fig. 5). The tumors had 5% to 25% of the neoplastic cells immunoreactive. The staining was at the periphery of the cytoplasm, and omitting the counterstain its membrane-bound distribution was better outlined in most samples (Fig. 6). One sample of atypical carcinoid (no. 21) was focally immunoreactive for sst2, despite negative RT-PCR findings. Conversely, sample no. 26 was immunohistochemistry negative and RT-PCR positive. The antiserum anti-sst2A (code K230) gave positive signal in 19 of 25 samples, in 5% to 60% of the neoplastic cell population (Fig. 7). The location of the staining was at the membrane level associated with a weak cytoplasmic reactivity. The same pattern was seen in positive controls, e.g., pancreatic islets (Fig. 7, inset). Two samples (nos. 19 and 26) were negative in spite of a positive RT-PCR signal. Two other tumors (nos. 21 and 22), apparently devoid of sst2 mRNA, showed a small percentage of immunoreactive cells. Incidentally, one of these latter samples (no. 21) was also immunoreactive with monoclonal antibody 10G4 (Table 2).



**FIG. 5.** sample no. 25 (small cell carcinoma). Absence of immunoreactivity for *ss12* with the monoclonal 10G4. This sample was also negative by reverse transcriptase polymerase chain reaction and in situ hybridization. (Immunoperoxidase in a formalin-fixed paraffin-embedded sample. Nuclei slightly counterstained with hemalum.) Bar: 45  $\mu$ m.



**FIG. 6.** sample no. 16 (typical carcinoid). Immunohistochemical detection of sst2 by means of monoclonal antibody 10C. The neoplastic cells have a peripheral cytoplasmic staining and membrane positivity in some cells, whereas the peribronchial gland adjacent to the tumor is unreactive. (Immunoperoxidase in a formalin-fixed paraffin-embedded sample. Nuclei slightly counterstained with hemalum.) Bar: 45  $\mu$ m. The membrane-bound distribution of the immunostaining is b

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outlined in a parallel section stained for monoclonal antibody 10G4 omitting nuclear counterstain (inset).

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**FIG. 7.** Same sample as in [Fig. 6](#). Immunohistochemical detection of sst2 by means of the polyclonal antibody K230. Th

immunostaining is more intense at the cell border (arrows), as observed with the monoclonal antibody. In the inset, a pancreatic islet, used as positive control, shows a predominant membrane-bound immunostaining of many neuroendocrine cells. (Immunoperoxidase in a formalin-fixed paraffin-embedded sample. Nuclei slightly counterstained with hemalum.) 45  $\mu$ m.

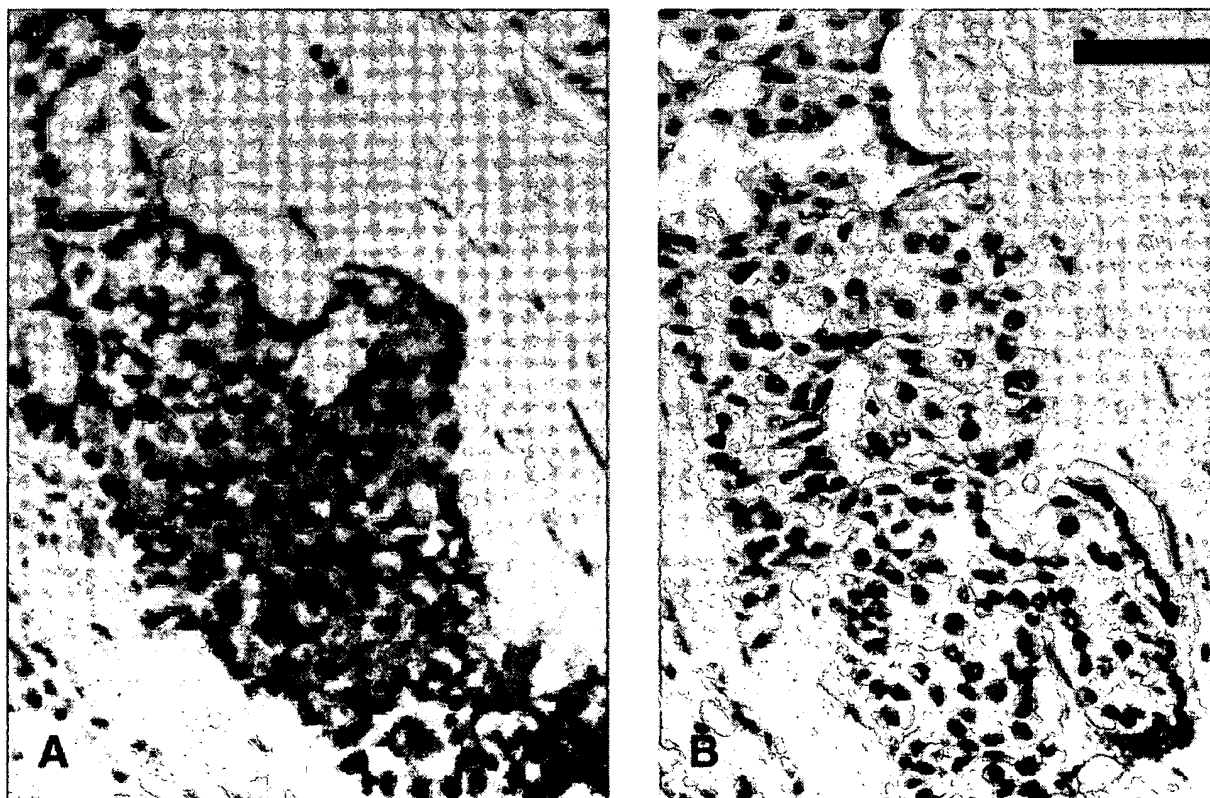
The five control samples positive by RT-PCR were also reactive with the antibodies. The type of immunocytochemical location of sst2 receptors was similar to that described here, being a peripheral cytoplasmic staining present in 40% to 70% of neoplastic cells. A weak and focal staining was also observed in five of the remaining RT-PCR-negative samples, when the antibody K230 was used (but not when the monoclonal was employed).

Several cells in peritumoral tissues were occasionally stained. Ciliated cells of bronchial mucosa had a peripheral staining at the cilia border. Mucous glands were negative. Rare chondrocytes had a membrane staining. The wall of peritumoral as well as of occasional distant vessels was stained at the endothelium level and in occasional smooth muscle cells.

The reactivity of both antibodies was abolished in serial sections when the reagents were preabsorbed with the respective synthetic peptides, but not when an unrelated peptide was used. The peritumoral bronchial mucosa had a focal staining of ciliated cells with both antibodies. This reactivity disappeared when the preabsorbed antibody was applied.

### In Situ Hybridization <sup>TOP</sup>

Eight of 12 samples stained by ISH were positive for sst2 mRNA. The mRNA was present in a percentage of cells (ranging from 10% to 40%) and gave a weak signal (Fig. 8), despite the amplification provided by the tyramide-based procedure. The background level was minimal using diluted biotinylated tyramide. Control sections stained with sense probe or an unrelated probe, or omitting the probe, were consistently negative.



**FIG. 8.** sample no. 11 (typical carcinoid). In situ hybridization (ISH) for sst2 mRNA shows a weak cytoplasmic staining in most tumor cells. An ISH performed with an unrelated probe was negative in a serial section of the same tumor (B). 1



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sample was strongly positive by reverse transcriptase polymerase chain reaction for sst2 mRNA and by immunohistochemistry. (Nonradioactive ISH revealed by peroxidase and diaminobenzidine, as substrate. Nuclei counterstained with hemalum.) Bar: 75 µm.

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## Clinical Data TOP

Clinicopathologic data are summarized in [Table 2](#). At follow-up, the majority of patients with typical carcinoids are free from disease 1 to 11 years after surgery. Two patients are alive with stable metastatic disease. Patients affected by atypical carcinoids had disease progression in one third of samples. Finally, patients with SCC had fatal outcomes within 1 year from diagnosis (except the recent sample). Eight patients had preoperative octreotide scintigraphy performed at the time of diagnosis. All patients had positive octreoscan findings, and, in these patients, also the tumor was positive by RT-PCR and immunohistochemistry or ISH. In addition, three of these patients received octreotide therapy administered at the time of tumor recurrence or metastatic spread. Stable disease is recorded at follow-up more than 5 years after diagnosis.

## Correlations TOP

Overall, complete overlapping (i.e., RT-PCR, ISH, and immunohistochemistry with two antibodies) between sst2 gene and protein expression was obtained in 21 of 25 samples (84%) and between RT-PCR results and immunohistochemical findings with at least one of the antibodies in 24 of 25 samples (96%). The monoclonal antibody 10G4 looked highly sensitive, being able to stain all but one sample (no. 26) (95%) positive for sst2 mRNA by RT-PCR. Sst2 expression, at mRNA as well as at protein levels, was reduced in high grade tumors, with SCCs being weakly positive in only two of five samples. Decreasing expression of sst2 appears to correlate with high tumor grade and elevated proliferative activity, but not with other parameters such sex, age, or tumor size.

## DISCUSSION TOP

In this study, the presence of sst2 mRNA has been demonstrated in a series of resected neuroendocrine tumors of the lung by means of RT-PCR and confirmed by a sensitive nonradioactive tyramide-based ISH procedure and by immunohistochemistry with anti-sst2 antibodies. Samples of both carcinoid tumors and SCCs were sst2 positive, although a reduced or absent signal was observed in poorly differentiated (small-cell) carcinomas. This is the first study of sst2 expression in a relatively large series of neuroendocrine tumors of the lung. Single samples of human carcinoids and SCCs (including cell lines of the latter) had previously been analyzed and found to express sst2 ([7,15,30,32,33,39,42](#)). Several methods have been used to detect these receptors and partially overlapping results were obtained.

In the present study, the expression of high amounts of sst2 mRNA was confirmed in well to moderately differentiated neuroendocrine tumors, in agreement with the results obtained by Reubi et al. ([32](#)) by means of radioactive ISH. The presence of sst2 mRNA in SCC had never been reported in human specimens, except for two samples included in Reubi et al.'s series ([32](#)). Although the data on cell lines support the observation that SCCs contain sst2 ([42](#)), slightly discrepant results were found in some of samples described here. Unfortunately, SCCs are rarely operated on, and therefore it is difficult to collect a large number of surgical specimens. The five samples studied in the current series by means of RT-PCR had a low amount (two samples) or absent (three samples) sst2 mRNA. This could be the result of the extensive necrosis commonly present in such tumor types. However, because care was taken to freeze fragments that were macroscopically devoid of necrotic areas, a more likely hypothesis is that sst2 expression is reduced in poorly differentiated tumors. Recently, Reisinger et al. ([29](#)) showed that the uptake of somatostatin analogues in patients with SCC undergoing chemotherapy is significantly lower, and therapeutic external factors may affect the receptor status of individual tumors. In addition, the uptake of somatostatin analogues in metastatic deposits of SCC has been shown to be low or absent ([29,2](#)). The present findings suggest that the sst2 mRNA content is related to the degree of tumor differentiation. These data must be confirmed in larger series of nonneuroendocrine tumors to ascertain whether the observed loss or decrease of sst2 expression in neuroendocrine tumors is a common event linked to neoplastic dedifferentiation. In addition, further studies are needed to assess the functionality of such receptors, by comparing the profile of sst2 expression in tumor tissues with binding assays employing labeled somatostatin and with the clinical response to diagnostic and therapeutic administration of somatostatin analogues.

To this purpose, several investigators have demonstrated a correlation between clinical imaging or response to somatostatin analogue treatment and sst2 mRNA content in single samples of carcinoid tumors ([15,22](#)). Northern blotting and ISH were the techniques used for sst2 mRNA identification. This kind of correlation is useful for selecting patients for somatostatin analogue treatment, although the demonstration of receptor mRNA in a cell does not imply per se that the receptor is fully functional.

The present study relied on a highly sensitive technique, RT-PCR, to identify all samples bearing even small amounts of sst2 mRNA. Indeed, in a previous study, single samples exhibiting octreotide-binding sites had no demonstrable sst2 mRNA by means of ISH, possibly due to the low sensitivity of the ISH procedure (34). The RT-PCR has shown sst2 mRNA transcripts in the majority of samples here studied. Only four samples were negative, all belonging to poorly differentiated high grade tumors, which usually follow an aggressive course. A decrease of sst2 mRNA expression in association with neuroendocrine tumor dedifferentiation had also been reported in neuroblastomas (37). In the above report, as well as in the current study, samples having an unfavorable prognosis were found to contain a relatively low amount of sst2 mRNA, as compared with well-differentiated tumors.

In the current sample series, eight samples were investigated before surgery with radiolabeled octreotide. Despite the low figures, all the samples positive at the diagnostic procedure had a strong RT-PCR signal for sst2 mRNA. Three of eight patients were also responsive to octreotide treatment administered at the time of relapse or metastatic spread. More extensive correlative clinicopathologic studies on the sst status are needed to better define the tissue distribution of somatostatin binding sites and their potential clinical role in the treatment of patients.

Sst2 evaluation by means of ISH (14,32) or RT-PCR (26,37) is a highly sensitive and reliable procedure. Unfortunately, these techniques have limitations because frozen tissue is needed for some of them, and radioactive material or costly and time-consuming methods are necessary for others. Immunohistochemical analysis of sst2 by means of specific antibodies represents an ideal, cheap, and rapid alternative, easily applicable to archival material. For these reasons, several investigators have raised polyclonal antibodies specific for sst (8,10,15,17,18). In the current study, tested tumor fragments adjacent to those snap frozen for RT-PCR analysis were tested with a polyclonal antibody against a C-terminal portion of the sst2A splice variant (35,36). In addition, a monoclonal antibody was produced in the Department of Pathology (University of Turin) against an N-terminal sequence of the human sst2. This antibody was the first monoclonal developed against sst2 and was shown to be highly specific for sst2 in Western blot and immunohistochemical analysis. Both the monoclonal and the polyclonal antibodies specifically reacted with all samples also positive by RT-PCR (with minor discrepancies in two samples, likely due to tumor heterogeneity). The observed correlation between RT-PCR and immunohistochemistry indicates that the latter may be a reliable diagnostic tool and may allow immunohistochemical investigation for sst2 even in small biopsy samples. This in turn may enable a rapid screening of sst2-positive tumors for medical treatment with somatostatin analogues.

Having confirmed in a relatively large series that the vast majority of neuroendocrine tumors of the lung contain variable amounts of sst2 mRNA, a final comment is deserved for sst2 expression in nonneuroendocrine lung carcinomas. No data have been reported thus far in the literature concerning normal human lung, although in the present study some bronchial cells of peritumoral parenchyma were positive for sst2 when immunohistochemical analysis was performed with either antibody. The staining was specific because it was abolished using preabsorbed antibodies. Therefore, it is likely that normal human lung tissue contains sst2. This might be confirmed by alternative techniques (e.g., Western blot, RT-PCR). However, in situ morphologic procedures, such as those employed here, have definite advantages. In fact, the lung is rich in vessels, and in several tissues (either in tumoral or in inflammatory-reactive conditions) the vessels were recently shown to contain sst (6).

A low expression of sst2 was found in 25% of lung carcinomas of nonneuroendocrine type investigated in the present study by means of RT-PCR. Therefore, sst type 2, at least, does not appear to be extensively expressed in nonneuroendocrine carcinomas of the lung. However, because two tumors in the control group (a squamous carcinoma and an adenocarcinoma, respectively) had positive octreotide scintigraphy, but no sst2 mRNA, it is plausible that a heterogeneous distribution of sst occurs in nonneuroendocrine lung tumors. Other receptor types may be expressed in these tumors and may be responsible for the positive results in diagnostic testing. Because sst5 is also known to bind somatostatin analogues, such as octreotide, with high affinity (24), the expression of this receptor type will be investigated in future studies.

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## REFERENCES TOP

1. Abbona GC, Papotti M, Viberti L, Macrì L, Stella A, Bussolati G. Chromogranin A gene expression in non-small cell lung carcinomas. *J Pathol* 1998; 186:1-6.  
[Context Link]
2. Berenger N, Moretti JL, Boaziz C, Vigneron N, Morere JF, Breau JL. Somatostatin receptor imaging in small cell



lung cancer. *Eur J Cancer* 1996; 32:1429-31.

[Context Link]

3. Bussolati G, Gugliotta P, Volante M, Pace M, Papotti M. Retrieved endogenous biotin: a novel marker and potential pitfall in diagnostic immunohistochemistry. *Histopathology* 1997; 31:400-7.

[Fulltext Link] [CrossRef] [Context Link]

4. Capella C, Heitz PU, Hofler H, Solcia E, Kloppel G. Revised classification of neuroendocrine tumors of the lung, pancreas and gut. *Virchows Arch* 1995; 425:547-60.

[Context Link]

5. Chirgwin JM, Przybyla AE, Mac Donald RJ, Rutter W. Isolation of biologically active RNA from sources enriched in ribonuclease. *Biochemistry* 1979; 18:5294-7.

[Context Link]

6. Denzler B, Reubi JC. Expression of somatostatin receptors in peritumoral veins of human tumors. *Cancer* 1999; 85:189-98.

[Context Link]

7. Fujita T, Yamaji Y, Sato M, Murao K, Takahara J. Gene expression of somatostatin receptor subtypes, SSTR1 and SSTR2, in human lung cancer cell lines. *Life Sci* 1994; 55:1797-806.

[CrossRef] [Context Link]

8. Gu WZ, Schonbrunn A. Coupling specificity between somatostatin receptor sst2A and G proteins: isolation of the receptor-G protein complex with a receptor antibody. *Mol Endocrinol* 1997; 11:527-37.

[CrossRef] [Context Link]

9. Gussow D, Rein R, Ginjaar I, Hochstenbach F, Seemann G, Kottman A, Ploegh HL. The human beta 2-microglobulin gene: primary structure and definition of the transcriptional unit. *J Immunol* 1987; 139:3132-8.

[Medline Link] [Context Link]

10. Helboe L, Moller M, Norregaard L, Schiodt M, Stidsen CE. Development of selective antibodies against the human somatostatin receptor subtypes sst1-sst5. *Brain Res Mol Brain Res* 1997; 49:82-8.

[CrossRef] [Context Link]

11. Helman IJ, Ahn TG, Levine MA, et al. Molecular cloning and primary structure of human chromogranin A (secretory protein I) cDNA. *J Biol Chem* 1988; 263:11559-63.

[Medline Link] [Context Link]

12. Hofland LJ, Liu Q, Van Koetsveld PM, et al. Immunohistochemical detection of somatostatin receptor subtypes sst1 and sst2A in human somatostatin receptor positive tumors. *J Clin Endocrinol Metab* 1999; 84:775-80.

[CrossRef] [Context Link]

13. Hsu SM, Raine L, Fanger H. Use of avidine-peroxidase complex (ABC) in immunoperoxidase techniques: comparison between ABC and unlabelled antibody (PAP) procedures. *J Histochem Cytochem* 1981; 29:577-80.

[Medline Link] [Context Link]

14. Janson ET, Gobl A, Kalkne KM, Oberg K. A comparison between the efficacy of somatostatin receptor scintigraphy and that of in situ hybridization for somatostatin receptor subtype 2 messenger RNA to predict therapeutic outcome in carcinoid patients. *Cancer Res* 1996; 56:2561-5.

[Context Link]

15. Janson ET, Stridsberg M, Gobl A, Westlin JE, Oberg K. Determination of somatostatin receptors subtype 2 in carcinoid tumors by immunohistochemical investigation with somatostatin receptor subtype 2 antibodies. *Cancer Res* 1998; 58:2375-8.

[Context Link]

16. Kerstens HMJ, Poddighe PJ, Hanselaar AGJM. A novel in situ hybridization signal amplification method based on the deposition of biotinylated tyramine. *J Histochem Cytochem* 1995; 43:347-52.

[Context Link]

17. Krisch B, Feindt J, Mentlein R. Immunoelectronmicroscopic analysis of the ligand-induced internalization of the somatostatin receptor subtype 2 in cultured human glioma cells. *J Histochem Cytochem* 1998; 46:1233-42.

[Context Link]

18. Kumar U, Laird D, Srikant CB, Escher E, Patel YC. Expression of the five somatostatin receptors (SSTR1-5) subtypes in rat pituitary somatotrophs: quantitative analysis by double-label immunofluorescence confocal microscopy. *Endocrinology* 1997; 138:4473-6.

[CrossRef] [Context Link]

19. Kwok S, Higuchi R. Avoiding false positives with PCR. *Nature* 1989; 339:237-8.

[Context Link]

20. Lamberts SWJ, Hofland LJ, Koetsveld PM, Reubi JC, Bruining HA, Bakker WH, Krenning EP. Parallel in vivo and in vitro detection of functional somatostatin receptors in human endocrine pancreatic tumors: consequences with regard to diagnosis, localization and therapy. *J Clin Endocrinol Metab* 1990; 71:566-74.

[Medline Link] [Context Link]

21. Malavasi F, Funaro A, Bellone G, et al. Functional and molecular characterization by the CB04 monoclonal antibody of a cell surface structure exerting C3-complement receptor activity. *J Clin Immunol* 1985; 5:412-20.

[Medline Link] [CrossRef] [Context Link]

22. Nilsson O, Kolby L, Wängberg B, et al. Comparative studies on the expression of somatostatin receptor subtypes, outcome of octreotide scintigraphy and response to octreotide treatment in patients with carcinoid tumours. *Br J Cancer* 1998; 77:632-7.

[Context Link]

23. Pagani A, Forni M, Tonini GP, Papotti M, Bussolati G. Expression of members of the chromogranin family in primary neuroblastomas. *Diagn Mol Pathol* 1992; 1:16-24.

[Context Link]

24. Panetta R, Greenwood MT, Warszynska A, et al. Molecular cloning, functional characterization and chromosomal localization of a human somatostatin receptor (somatostatin receptor type 5) with preferential affinity for somatostatin-28. *Mol Pharmacol* 1994; 45:417-27.

[Context Link]

25. Papotti M, Macri L, Bussolati G, Reubi JC. Correlative study on neuroendocrine differentiation and presence of somatostatin receptors in breast carcinomas. *Int J Cancer* 1989; 43:365-9.

[Medline Link] [Context Link]

26. Papotti M, Macri L, Pagani A, Aloï F, Bussolati G. Quantitation of somatostatin receptor type 2 in neuroendocrine (Merkel cell) carcinoma of the skin by competitive RT-PCR. *Endocr Pathol* 1999; 10:1-10.

[Context Link]

27. Patel YC, Panetta R, Escher E, Greenwood M, Srikant CB. Expression of multiple somatostatin receptor genes in AtT-20 cells: evidence for a novel somatostatin-28 selective receptor subtype. *J Biol Chem* 1994; 269:1506-9.

[Context Link]

28. Pearson WR, Lipman DJ. Improved tools for biological sequence comparison. *Proc Natl Acad Sci U S A* 1988; 85:2444-8.

[Medline Link] [CrossRef] [Context Link]

29. Reisinger I, Bohuslavtzi KH, Brenner W, et al. Somatostatin receptor scintigraphy in small-cell lung cancer: results of a multicenter study. *J Nucl Med* 1998; 39:224-7.

[Context Link]

30. Reubi JC, Kappeler A, Wsær B, Laissue J, Hipkin RW, Schonbrunn A. Immunohistochemical localization of somatostatin receptors sst2A in human tumors. *Am J Pathol* 1998; 153:233-45.

[Context Link]

31. Reubi JC, Maurer R, von Werder K, Torhorst J, Klijn JGM, Lamberts SWJ. Somatostatin receptors in human endocrine tumors. *Cancer Res* 1987; 47:551-8.

[Medline Link] [Context Link]

32. Reubi JC, Schaer JC, Waser B, Mengod G. Expression and localization of somatostatin receptors SSTR1, SSTR2, SSTR3 messenger RNA in primary human tumors using in situ hybridisation. *Cancer Res* 1994; 54:3455-9.

[Context Link]

33. Reubi JC, Waser B, Sheppard M, Macaulay V. Somatostatin receptors are present in small-cell but not in non-small-cell primary lung carcinomas: relationship to EGF receptors. *Int J Cancer* 1990; 45:269-74.

[Medline Link] [Context Link]

34. Schaer JC, Waser B, Mengod G, Reubi JC. Somatostatin receptors subtypes sst1, sst2, sst3 and sst5 expression in human pituitary, gastroentero-pancreatic and mammary tumors: comparison of mRNA analysis with receptor autoradiography. *Int J Cancer* 1997; 70:530-7.

[CrossRef] [Context Link]

35. Schindler M, Holloway S, Humphrey PPA, Waldvogel H, Faull RLM, Berger W, Emson PC. Localization of the somatostatin sst2a receptor in human cerebral cortex, hippocampus and cerebellum. *Neuroreport* 1998; 9:521-5.

[Fulltext Link] [CrossRef] [Context Link]

36. Schindler M, Sellers LA, Humphrey PPA, Emson PC. Immunohistochemical localization of the somatostatin sst2(A) receptor in the rat brain and spinal cord. *Neuroscience* 1997; 76:225-40.

[CrossRef] [Context Link]

37. Sestini R, Orlando C, Peri A, et al. Quantification of somatostatin receptor type 2 gene expression in neuroblastoma cell lines and primary tumors using competitive reverse transcription-polymerase chain reaction. *Clin Cancer Res* 1996; 2:1757-65.

[Context Link]

38. Speel EJM, Saremaslani P, Roth J, Hopman AHN, Komminoth P. Improved mRNA in situ hybridization on formaldehyde-fixed and paraffin-embedded tissue using signal amplification with different haptenized tyramides. *Histochem Cell Biol* 1998; 420:1-7.

[Context Link]

39. Taylor JE, Theveniau MA, Bashirzadeh R, Reisine T, Eden PA. Detection of somatostatin receptor subtype 2 (SSTR2) in established tumors and tumor cell lines: evidence for sst2 heterogeneity. *Peptides* 1994; 15:1229-36.

[CrossRef] [Context Link]

40. Travis WD, Gal AA, Colby TV, Klimstra DS, Falk R, Koss MN. Reproducibility of neuroendocrine lung tumor classification. Hum Pathol 1998; 29:272-9.

[Context Link]

41. Yamada Y, Post SR, Wang K, Tager H, Bell GI, Seino S. Cloning and functional characterization of a family of human and mouse somatostatin receptors expressed in brain, gastrointestinal tract and kidney. Biochemistry 1992; 89:251-5.

[Context Link]

42. Zhang CY, Yokogoshi Y, Yoshimoto K, Fujinaka Y, Matsumoto K, Saito S. Point mutation of the somatostatin receptor 2 gene in the human small cell lung cancer cell line COR-L103. Biochem Biophys Res Commun 1995; 210:805-15.

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